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13. ABSTRACT *(Maximum 200 Words)*

The purpose of work in year 03 of this study was to continue to define the in vitro growth characteristics of stable cell lines of human breast carcinoma cells (developed in year 01 and continuing into year 02) that synthesized varying levels of plasminogen activator inhibitor type-1 (Pai-1) as a result of transfection with expression vectors bearing PAI-1 cDNA inserts cloned in the sense and antisense orientations. A complete panel of 32 such genetically-engineered epithelial cell lines was created and each of these were assessed with regard to their ability to (1) locomote across a planar surface following scrape-injury of confluent monolayers (directed cell migration) and (2) exhibit invasive growth behavior in three-dimensional motility chamber assays. PAI-1 expression was required for epithelial cell migration in directed assays since antisense targeting effectively suppressed induced cell locomotion. A unique expression vector consisting of a PAI-1-GFP chimeric protein driven by PAI-1 promoter sequences was used to conclusively demonstrate PAI-1 deposition into cellular migration tracks. A "window" of PAI-1 expression was found to be necessary to support optimal breast cancer locomotion and invasive migration. Work in the final year will test this hypothesis in vivo.

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Introduction

Cancer invasion and metastasis are complex processes in which controlled and focalized extracellular matrix (ECM) degradation facilitates cellular migratory activity. ECM turnover is regulated temporally and spatially by the concerted action of several interacting and cascading proteolytic systems including, most prominantly, the generation of plasmin by the urokinase (uPA)-dependent pathway of plasminogen activation (Dano et al., 1994; Andreasen et al., 1997). Plasmin, in turn, degrades the ECM directly as well as indirectly by activating latent metallo proteinases (e.g; MMP1, 2, 9) (Dano et al., 1987; Vaheri et al., 1990; Pollanen et al., 1991). Within this context, plasminogen activator inhibitor type-1 (PAI-1) plays a primary role as a negative regulator of the plasmin-based pericellular proteolytic cascade by complexing to, and inhibiting the catalytic activity of, free as well as receptor-bound uPA (Blasi et al., 1987; Andreasen et al., 1997) **(Figure 1)**. Studies in mice genetically engineered to be deficient in one or more elements in the plasmin activation cascade have confirmed the importance of uPA and plasmin in cell migration (Romer et al., 1996; Carmeliet et al., 1997). Cell type-specific synthesis and subcellular targeting of PAI-1 and uPA appear to be important considerations in modulation of the pericellular proteolytic balance. Temporal changes in the expression, focalization, and/or relative activity of this protease/inhibitor pair may influence cell migration either as a direct consequence of ECM barrier proteolysis or by modulating cellular adhesive interactions with the ECM (Ciambrone and McKeown-Longo, 1990; Blasi, 1996, 1997; Carmeliet et al., 1997) **(Figure 2 and 3)**. This has direct relevance to outcomes in human breast cancer. Clinical studies have demonstrated that, in general, elevated tumor levels of uPA, the uPA receptor, and PAI-1 are conducive to tumor metastasis and associated with poor disease prognosis (Schmidt et al., 1992; Duffy, 1992, 1996; Pedersen et al., 1994). The role of PAI-1 as a determinant in aggressive growth behavior is particularly important in breast carcinoma (Costantini et al., 1996; Gandolfo et al., 1996; Mayerhofer et al., 1996; Fersis et al., 1996; Torre and Fulco, 1996; Foekens et al., 1995). The purpose of this work, therefore, is to utilize a molecular genetic approach to manipulate levels of PAI-1 expression in human breast carcinoma cells to (1) generate panels of genetically-engineered human breast cancer cells which vary in levels of PAI-1 expression/synthesis, (2) assess the *in vitro* growth characteristics of these cells (specifically as regards invasive behavior), and (3) evaluate the usefulness of the PAI-1 gene as an *in vivo* therapeutic target. To do this, we have constructed vectors bearing a full-length PAI-1 cDNA. Expression of this cDNA, cloned in both sense and antisense orientations, is under control of various, tandemly-linked, copies of MYC-responsive E-box promoter sequences to take advantage of the endogenous breast cancer MYC protein as a means to regulate transcriptional strength. Such utilization of tumor-associated anomalies in transcription factor (i.e., MYC) expression to direct genetic-based intervention therapies is a new approach to cancer treatment. This strategy, moreover, addresses the more aggressive breast tumor cell type, the highly proteolytically active potentially metastatic cells, for specific therapy. Our approach takes advantage of the amplified MYC expression typical of breast carcinoma to transactivate transfected vectors resulting in high level sense and antisense PAI-1 transcript production. These studies will provide information critical to the eventual design of tumor type-appropriate targetable delivery systems for genetic therapy of breast cancer.

Body of Report

We had originally hypothesized that over-expression of PAI-1 by malignant epithelial cells was an important aspect in the progression of a relatively indolent localized tumor to an invasive,

highly metastatic, phenotype. Based on data summarized in the **Introduction**, our working model is that temporal changes in the expression, focalization, and or relative activity of urokinase PA/PAI-1 may influence cellular migration and invasive traits either as a direct consequence of ECM barrier proteolysis or by modulating cellular adhesive interactions (via cycles of adhesion/de-adhesion) with the supporting matrix **(Figure 1-3)**. This model is consistent with recent findings indicating that PAI-1, specific integrins, and urokinase PA function coordinately to regulate adhesive events important in the control of cell movement. Our recently published observations (Providence et al., 2000; Kutz et al., 2001) provide initial experimental verification of this concept using a relatively simple culture system of induced epithelial cell locomotion over a planar substrate. Two aspects of this work are directly relevant to the working hypothesis of the present grant. Epithelial cells exhibit a location-specific distribution of PAI-1 expressing cells (i.e., only locomoting cells express this SERPIN) and down-regulation of PAI-1 synthesis and its matrix deposition in epithelial cells stably transfected with a PAI-1 antisense expression vector significantly impaired cell migration. Most importantly, it was possible to "rescue" a PAI-1 functionally-null motility-deficient epithelial cell line by transfection with a PAI-1 expression vector (Providence et al., 2000). Rescued cells expressed approximately normal levels of PAI-1 and efficiently locomoted in response to a denuding stimulus. Moreover, we most recently have constructed a PAI-1-GFP expression vector regulated by upstream CMV promoter sequences **(Figure 4)**. This vector was used to confirm deposition of PAI-1 into cellular migration tracks. We have taken advantage of the availability of this vector to engineer additional breast cancer lines that express PAI-1-GFP. This will allow us to "track" highly aggressive human breast cancer cells following transplantation into nude mice and provide a sensitve tag to detect micrometastases..

These results are consistent with the **Specific Aims** of this proposal which were (1) to generate transfectants of human breast carcinoma cells that vary in PAI-1 expression at both the mRNA and protein levels and to characterize the *in vitro* growth traits of these genetically-engineered cells, (2) to assess the *in vivo* growth characteristics of human breast carcinoma cells engineered to express different levels of PAI-1 using the results obtained in **Aim 1** to identify the most important candidate clones to evaluate, and (3) to initiate studies designed to assess the targetability of *in vivo*-implanted human breast tumor cells with potential therapeutically-relevant vectors as concluded from results obtained on work directed to **Aims 1 and 2**.

To achieve these aims, the goals in **Task 1** in the originally proposed **Statement of Work** were as follows:

Task 1: To assess the effects of vector-directed PAI-1 expression on *in vitro* growth traits of human breast carcinoma cells.

(a) develop a panel of transfectant MCF-7 (estrogen responsive) and MDA-MB-231 (estrogen receptor negative) breast carcinoma cells which express differing levels of vector-driven PAI-1 mRNA and protein.
(b) perform assays to assess the *in vitro* growth characteristics of the individual transfectant cell lines.
(c) correlate levels of PAI-1 gene expression for each transfectant cell line with specific *in vitro* growth traits.

We have completed work designed to address **Task 1.** A total of 32 different stable transfectant breast carcinoma cell lines were developed; 17 MCF-7 derived lines bearing a positive sense PAI-1 expression vector (either Rc/CMVPAI or Rc/E-BoxPAI) and in which PAI-1 mRNA levels varied from 0.8±0.1 to 49.6±9.0 fold over MCF-7 parental or vector without insert controls and 15 MDA-MB-231 antisense PAI-1 (either Rc/CMVIAP or Rc/E-BoxIAP) vector-derived lines in which PAI-1 mRNA abundance was down-regulated by 11 to 92% compared to exponentially growing parental controls or cells transfected with vector without insert. A panel of 6 transfectant lines (for both the MCF-7 and MDA-MG-231 derived cell types) was selected for analysis of *in vitro* growth traits based on levels of PAI-1 expression significantly different from either of the parental strains. A quantitative analysis of these growth traits is summarized in **Table 1**. As a result of these analyses, it is anticipated that these same parental and transfectant cell lines will form the panel for the *in vivo* testing phase of this study (**as detailed in the original proposal**). In addition, we have generated 4 additional transfectant cell lines in the MCF-7 cellular background which provide the unique quality of estrogen-inducibility of MYC expression thereby allowing for controlled vector-driven PAI-1 expression for continued growth trait assessments. Each of these clonal derivatives express PAI-1 sense transcripts under control of MYC-responsive E box modules; PAI-1 synthesis by these 4 lines is a stable characteristic with levels of expression 5- to 9-fold greater than the original 7/EBPAI-D isolate (**Table 1**). Chemokinetic and chemotactic evaluations of these cells is presently ongoing. A most important development during this last year was our ability to construct a chimeric expression vector in which a PAI-1-GFP fusion insert was cloned under control of CMV promoter sequences (**Figure 4**). We have successfully created stable transfectants of MCF-7 cells with this vector that express, process and transport PAI-1-GFP in a manner identical to the endogenous PAI-1 protein. These genetically-"tagged" cells can be tracked in vivo for unambiguous evaluation of micrometastatic deposits

Key Research Accomplishments

A panel of transfectant human breast carcinoma cell lines (derived from both MCF-7 and MDA-MB-231 parental stocks) was developed that varied in vector-driven synthesis of PAI-1 mRNA and protein. These cell lines are suitable for conduct of all *in vitro* and *in vivo* growth assessments as originally proposed.

Comparisons between *in vitro* growth traits and PAI-1 expression as a function of cell growth activation indicated that MCF-7 cells were low to non-PAI-1 expressing regardless of proliferative stage (i.e., quiescent vs. cycling G1 vs. exponentially growing) whereas MDA-MB-231 cells expressed relatively high levels of PAI-1 mRNA/protein, particularly in serum-supplemented culture conditions. This differential in PAI-1 synthesis correlated with the low intrinsic motility (i.e., directed migration across denuded planar surfaces) of MCF-7 cells compared to the highly migratory phenotype of MDA-MB-231 carcinoma cells.

Extent of cell spreading on vitronectin-coated bacteriological culture dishes was approximately inversely related to the level of PAI-1 expressed by transfected MCF-7 cells (i.e., MCF-7 cells attached and spread onto vitronectin; the extent of cell spreading decreased with increasing vector-driven expression of PAI-1). There was no direct quantitative relationship between MCF-7 cell spreading on fibronectin and PAI-1 levels although high PAI-1-expressing MCF-7 cells were flatter on fibronectin-coated dishes than their low-PAI-1 expressing

counterparts. MDA-MB-231 parental cells that constitutively synthesized abundant PAI-1 mRNA/protein were highly motile and formed extensive membrane ruffles (indicative of a locomoting phenotype). PAI-1 antisense vector-directed down-regulation of PAI-1 synthesis in clonal isolates of MDA-MB-231 cells resulted in increased cell spreading (compared to parental controls), loss of membrane ruffling and decreased migration across a planar substrate.

Increased PAI-1 expression in PAI-1 sense vector MCF-7 transfectants resulted in an increase in random migration as well as augmenting both the chemokinetic and chemotactic index. PAI-1 expression was necessary for elaboration of the migratory/invasive phenotype in MDA-MB-231 cells as a vector-driven down-regulation of PAI-1 synthesis in this cell type reduced the fraction of cells capable of random, chemokinetic and chemotactic migration.

Four different clonal isolates of MCF-7 cells (stable transfectants) were generated using a PAI-1 vector in which the cDNA insert was expressed under control of MYC-responsive E box sequences.

Stable cell lines in the MCF-7 background were derived using a CMV promoter-driven PAI-1-GFP insert. These cells express, process, and secrete a PAI-1-GFP fusion protein that has biological characteristics identical to that of the endogenous PAI-1 protein. These cells will be important in evaluation of micrometastases

Reportable Outcomes

1. The following manuscripts, abstracts, and presentations directly resulted from support of this project by the Department of the Army under grant DAMD17-98-1-8015.

Providence KM, Kutz SM, Staiano-Coico L, Higgins PJ (2000) PAI-1 gene expression is regionally induced in wounded epithelial cell monolayers and required for injury repair. **Journal of Cellular Physiology** 182: 269-280.

Lee F, Goncalves J, Faughman K, Steiner MG, Pagan-Charry I, Chin B, Providence KP, Higgins PJ, Staiano-Coico L (2000) Targeted inhibition of wound-induced PAI-1 expression alters migration and differentiation in human epidermal keratinocytes. **Experimental Cell Research** 258: 245-253.

White LA, Bruzdzinski C, Kutz SM, Gelehrter TD, Higgins PJ (2000) Growth state-dependent binding of USF-1 to a proximal promoter E box element in the rat plasminogen activator inhibitor type-1 gene. **Experimental Cell Research** 260: 127-155.

Kutz SM, Providence, KM, Higgins, PJ (2001) Antisense targeting of c-*fos* transcripts inhibits serum- and TGF-β1-stimulated PAI-1 gene expression and directed motility in renal epithelial cells. **Cell Motility and the Cytoskeleton** 48, 163-174.

Higgins, P.J. (2001) Genetic perturbation of PAI-1 gene expression in human breast carcinoma cells alters growth factor-dependent migration and invasion. **Experimental Cell Research** (submitted).

Tang, J. and Higgins, P.J. (2001) Visualization of PAI-1-dependent human breast cancer cell motility using a PAI-1-GFP expression system. (in preparation).

Higgins PJ (2000) PAI-1 gene expression in breast carcinoma cells: implications for cellular migratory activity. (Abstract, Era of Hope Meeting, Atlanta, Georgia, June 8-12).

Higgins PJ (2000) "Molecular mechanisms underlying growth state-dependent PAI-1 gene expression". (Invited seminar, Stratton VA Medical Center, Albany, New York, Feb. 17).

Higgins PJ :(2000) "Genetic targeting of PAI-1 gene expression: consequences on cellular migration" (Invited seminar, David Axelrod Institute, Cancer & Endocrine Signaling Seminar Series, NY State Department of Health, Albany, New York, March 13).

2. Development of cell lines:

All MCF-7 and MDA-MB-231 cell lines and their transfectant derivatives will be maintained in Dr. Higgins' laboratory. These cells will be made available upon request to members of the scientific community engaged in breast cancer research.

Conclusions

The following is a summary of the conclusions of the present report and the implications of the results obtained.

PAI-1 expression levels in indolent breast carcinoma cells (e.g., MCF-7) are low relative to the abundant expression of PAI-1 mRNA/protein characteristic of highly aggressive and metastatic breast tumor cell types (e.g., MDA-MB-231). PAI-1 synthesis in MCF-7 cells, moreover, was uninfluenced by growth state whereas PAI-1 was highly expressed in the phenotypically aggressive MDA-MB-231 cell line suggesting that expression is linked to the biological behavior of a particular breast tumor rather than simply to cellular proliferation. These results suggest that assessments of PAI-1 synthesis in breast cancers may represent an important parameter for patient classification. Patients presenting with high PAI-1-positive primary breast tumors may benefit from more aggressive therapy and post-operative surveillance. Analysis of our transfectant tumor cell lines additionally indicates that high levels of PAI-1 expression clearly modulates cell-to-matrix protein attachment properties and facilitates the elaboration of an invasive phenotype. Our studies detailing the effect of molecular targeting of PAI-1 gene expression in epithelial cells on induced directional migration into scrape-denuded "wounds" (Providence et al., 2000) is consistent with the present findings implicating PAI-1 as an essential element in the migratory apparatus of breast carcinoma cells. Our ability to actually visualize a PAI-1-GFP fusion protein in the migration "trails" of transfected cells during the real time of cell movement reinforce our gene targeting findings. Collectively, these data support the original hypothesis that the PAI-1 gene represents a reasonable (and now clearly an accessible therapeutic target in the treatment and management of aggressive human breast cancers. Our work has also afforded an important opportunity to create genetically-engineered human breast cancer lines and targeted vector systems that we believe will be critical tools for future studies on gene therapy approaches to breast cancer management.

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APPENDIX

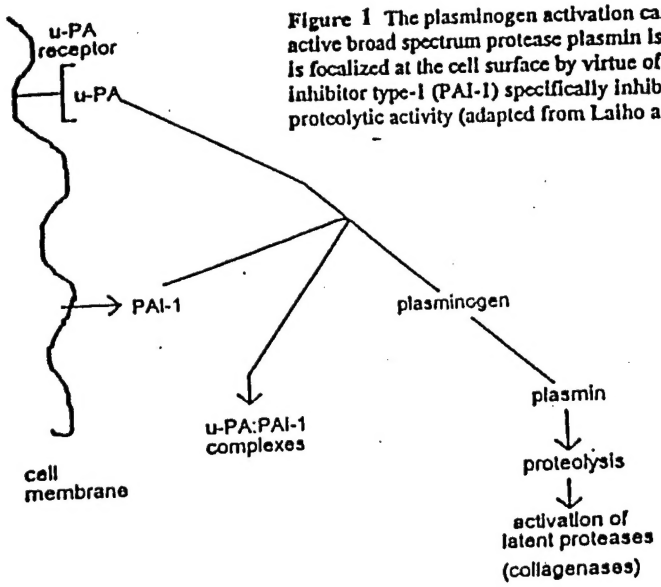

Figure 1 The plasminogen activation cascade. Conversion of the inactive zymogen plasminogen to the active broad spectrum protease plasmin is catalyzed by urokinase type plasminogen activator (u-PA) which is focalized at the cell surface by virtue of interaction with its specific receptor. Plasminogen activator inhibitor type-1 (PAI-1) specifically inhibits this reaction resulting in a net decrease in pericellular proteolytic activity (adapted from Laiho and Keski-Oja, 1989).

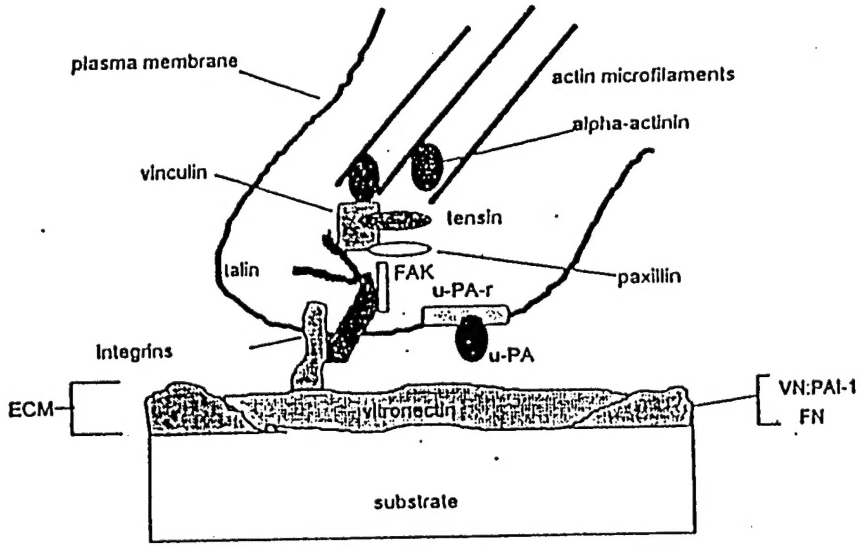

Figure 2 Idealized schematic illustrating localization of u-PA and PAI-1 at focal adhesion sites. The relationship between constituents of the focal contact and components of the plasmin-mediated proteolytic system demonstrate the potential importance of the plasmin-mediated proteolytic cascade as a regulator of cell-to-substrate adhesion. u-PA (associated with its receptor) and PAI-1 are localized to the extracellular face of the cell within and surrounding the focal contact, respectively (derived from Pollanen et al., 1991 and Ezzell, 1993).

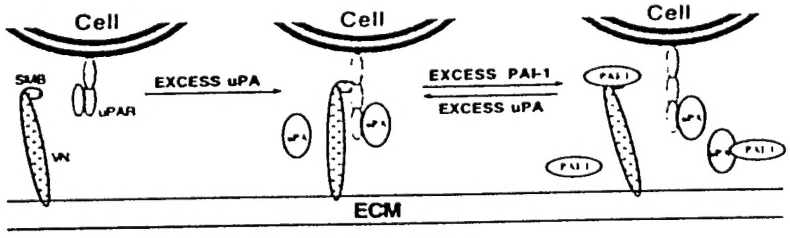

Figure 3 Model for the regulation of uPAR dependent cell adhesion and release by PAI-1 and uPA. (from Deng et al., 1996)

Table 1. Migratory characteristics of parental and transfectant human breast carcinoma cell lines.

Cell Line	Relative PAI-1 Expression	Migration Indices	
		Chemokinetic	Chemotactic
MCF-7	1.0	276±129	366±109
7/CMV	0.8±0.1	201±74	290±59
7/EBPAI-D	4±1	297±101	431±207
7/CMVPAI-E	12±3	432±88	716±244
7/CMVPAI-B	22±5	699±121	701±153
7/CMVPAI-K	30±6	748±97	1289±346
7/CMVPAI-H	49±9	1121±277	3348±601
MDA-MB-231	100	742±93	2392±316
231/CMV	108±11	891±76	2203±237
231/CMVIAP-6	89±20	525±43	1114±179
231/CMVIAP-9	64±7	312±44	700±191
231/EBIAP-15	40±9	285±59	513±105
231/EBIAP-3	17±6	171±22	327±98
231/EBIAP-21	8±3	143±6	202±53

Relative PAI-1 Expression for MCF-7 transfectants = fold increase in de novo synthesized PAI-1 protein relative to parental cells (set at 1.0).

Relative PAI-1 Expression for 231 transfectants = % decrease in de novo synthesized PAI-1 protein relative to parental cells (set at 100%).

Migration Indices for the chemokinetic (without attractant gradient) and chemotactic (with attractant gradient) indicates the number of cells migrating to the lower chamber of a micro-chemotaxis apparatus within 3 hours after seeding of 5×10^4 cells to the upper chamber (methodology detailed in the original application).

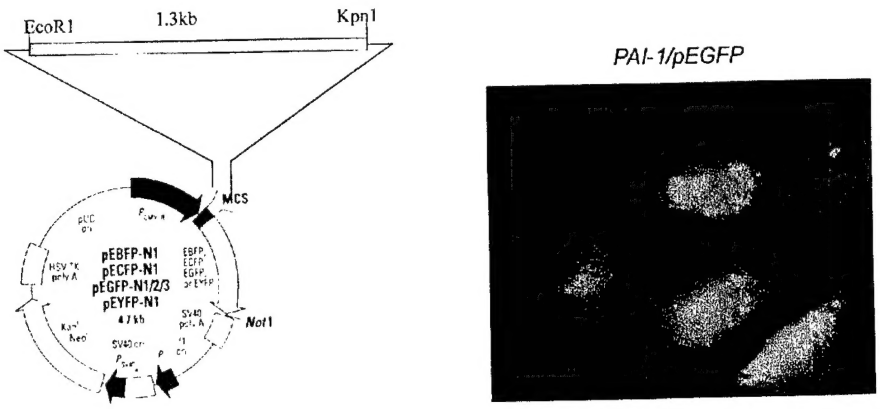

Figure 4. Details as to cloning of a 1.3 kb insert representing complete PAI-1 coding sequences into a GFP fusion vector (left panel). Upon transfection, this construct was effectively expressed as served as a "tag" for PAI-1-synthesizing cells.

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